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TITLE: Multiplexed Analysis of Circulating Prostate Tumor and Host Response Markers

PRINCIPAL INVESTIGATOR: Brian B. Haab, Ph.D.
Alan W. Partin, M.D., Ph.D.
Bin Sing Teh, M.D.

CONTRACTING ORGANIZATION: Van Andel Research Institute
Grand Rapids, MI 49503

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Brian B. Haab, Ph.D.
Alan W. Partin, M.D., Ph.D.
Bin Sing Teh, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Van Andel Research Institute
Grand Rapids, MI 49503

E-Mail: Brian.haab@vai.org

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13. ABSTRACT (Maximum 200 Words)

During the second year of this project we have made further strides toward the broad goals of this project to develop improved blood-based methods for prostate cancer diagnostics and to better understand the nature of the changes in the blood of prostate cancer patients. The first year of the project was used to lay some of the fundamental foundations for the later work: we answered valuable questions about the optimal strategies for using antibody microarrays, we better characterized the performance of the antibodies, and we significantly improved the experimental protocols. The work of the second year has built upon those developments and has resulted in several accomplishments. We further developed and improved the methods by instituting improved quality control for the antibodies, by developing an improved ability to run samples in high-throughput, and by identifying a better-performing microarray surface. We performed initial profiling studies on serum samples from prostate cancer patients and controls using antibody microarrays containing 96 or 91 antibodies. The preliminary analyses identified areas of the technology that needed improving or that required an alternate strategy. We have addressed and solved the potentially confounding influence of species-dependent, non-specific binding, and we are using the alternate strategy of sandwich immunoassays for a select set of low-abundance analytes. The early analyses also identified several interesting differences between the patient groups, which will be further investigated. Our improved methods are currently being used for comprehensive profiling studies of our serum sample sets, and the data from these studies will be analyzed to fulfill the goals of year 3.

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Principal Investigator: Haab, Brian B.

INTRODUCTION

The report contains a summary from the second year of work on our project to analyze the variation in protein composition in the sera of prostate cancer patients and controls. The broad goals of this project are to develop markers that aid in the specific diagnosis of prostate cancer and to gain insight into the functional significance of serum composition alterations that are associated with the disease. The project was divided into three tasks. Task 1 related to optimizing and validating the methods that would be used in Task 2. Most of Task 1 has been completed, with one part ongoing for practical reasons described in the report. The goal of Task 2 was to acquire profiles of a wide variety of proteins in serum samples from prostate cancer patients and controls. That task has been completed in initial data sets, yet we are continuing to collect data on the samples to bolster the data we already have. Task 3 concerned the bioinformatics analyses of the data to uncover relationships among the proteins and samples. Initial analyses have been performed, and further analyses will be conducted in year 3 as the data become available. Much more information about the performance of the assay has been gathered, which has led to improvements to the assays or alternate strategies in some cases. The improvements to the methods and the alternate strategies we are using will result in much more complete and valuable data than we otherwise would acquire. We expect very valuable information to be generated from the profiling experiments that are currently underway, and we are on target to complete all tasks at the end of year 3.

BODY

1. Summary of progress on Task 1

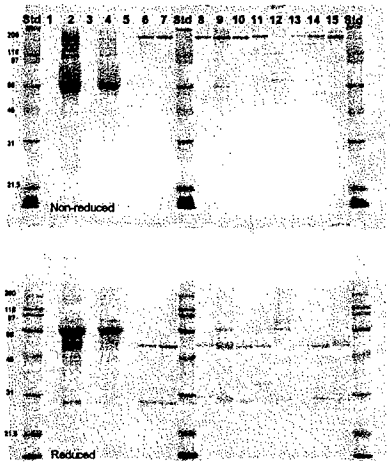
The purpose of the work in Task 1 was to determine the optimum conditions for taking microarray measurements for each of the antibodies. Most of the work of that task had been completed in year 1. In that year we gained a greater understanding of the requirements for setting optimal serum dilution factors and for measuring multiple serum proteins of varying concentrations with a single dilution. We found that our two-color competitive assay allowed us to accurately measure different proteins of widely varying concentrations, provided the concentration of each protein in the reference solution was approximately matched to the concentration of the corresponding protein in the sample solutions. Therefore, we could measure proteins in the mg/ml range and proteins in the ng/ml range using the same dilution factor and the same detection method. Our two-color rolling circle amplification (TC-RCA) method was preferable to non-amplified detection. We performed some additional work on Task 1 in year 2, as described below. The statement of work for Task 1 is below, with a detailed description of the progress on each point following.

Task 1. Collection of antibodies and characterization of their accuracy, specificity and linear range in the microarray assay using both direct fluorescent detection and rolling circle amplification, Months 1-9:

a. Collect antibodies from collaborators and vendors, purify IgG fraction if necessary, prepare in proper buffer and distribute in microtiter plates for printing.

b. Perform microarray experiments using each detection method on a set of 20 serum samples, in triplicate. Perform ELISA assays (if available) for each target protein on the same serum samples, in triplicate. Compare the ELISA and microarray results to assess the accuracy and linear range of each antibody using each detection method.
c. Perform microarray experiments using each detection method on dilutions of each of the 20 serum samples; assess the linearity and linear range of each antibody in each detection method.
d. Perform microarray experiments on selected serum samples with and without spiking in unlabeled antigen (if available) or antibody for each target protein. Evaluate the reduction of signal from the microarray to assess the specificity of antibody binding on the microarray.

Step a. This step was essentially finished in year 1. In year two, we added about seven new antibodies to the experiments. We added a quality control step for every antibody, in which every antibody gets analyzed on a reducing and non-reducing gel. This step allows us to confirm that each antibody is intact, is free from major impurities, and is close to the stated concentration. This procedure has resulted in the identification of non-intact antibodies and antibodies with high-concentration additives. An example of this quality control step is shown in Figure 1. In Figure 1, the antibody in lane 12 is suspect because of the presence of extra bands and weak bands at the correct molecular weight.



Lane	Antibody
1,3,5	Empty
2	VAI00335B Ascites
4	VAI00335B Protein A flow through
6	VAI00335B IgG Post-dialysis
7	VAI00335B.1 IgG Final anti-Gelsolin
8	VAI00271 anti-Thioredoxin
9	VAI00273.1 anti-Ceruloplasmin
10	VAI00274 anti-Alkaline Phosphatase
11	VAI00282B.1 anti-Alpha fetoprotein
12	VAI00346 anti-Amylase
13	VAI00351.1 anti-Serum amyloid A
14	VAI00382B.1 anti-CRP
15	VAI00416B anti-Cathepsin D

Figure 1. Gel confirmation of the integrity of antibodies to be spotted on arrays.

Step b. In year 1, we determined that the RCA detection method was just as accurate and precise as our direct labeling method. In addition, the linear range was similar, since we were using a competitive assay with a matched reference solution, so we were able to use RCA for the measurement of both high and low abundance proteins, provided the reference concentrations were somewhat matched. That condition is guaranteed by our procedure of using a pool of all the samples as the reference solution. Therefore this subtask was completed, and we elected to use the TC-RCA method at one dilution for our profiling experiments.

Step c. The purpose of this step was to determine if the linear range of detection was different between RCA detection and direct labeling, which then could necessitate the use of two detection methods for every sample—RCA for low-abundance proteins and direct labeling for higher abundance proteins. As described above, we learned that RCA could be used for both high and low abundance proteins because of the performance characteristics of the competitive assay. In year 1 we further explored the question of optimal dilution factor, and whether each sample should be run at multiple dilutions. We found a minimal advantage in running more than

one dilution with the competitive assay. Therefore, this step was completed in year 1, and we will use TC-RCA at one dilution for the initial profiling experiments.

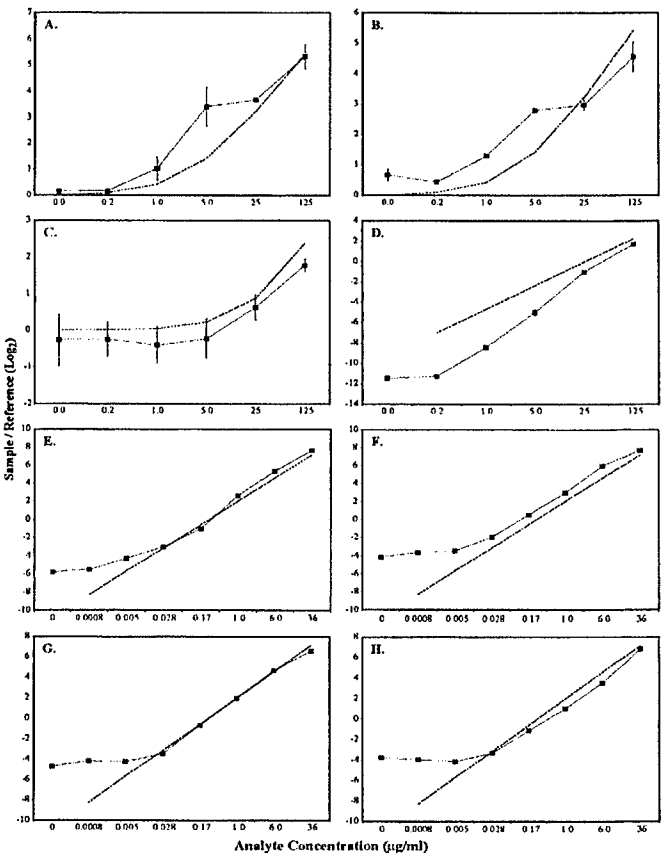


Figure 2. Antibody binding validation using analyte dilutions. The ratio (log_2) of sample-specific fluorescence to reference-specific fluorescence at a given antibody was plotted with respect to the concentration of the respective analyte. (A) Anti-CEA measuring purified CEA spiked into human serum. (B) Anti-lipase measuring purified lipase spiked into human serum. (C) Anti-complement C3 measuring purified complement C3 spiked into human serum. (D) Anti-complement C3 measuring purified complement C3 spiked into a BSA solution. (E) Anti-CRP (ab #2) measuring recombinant CRP spiked into a BSA solution. (F) Anti-hemoglobin measuring purified hemoglobin spiked into a BSA solution. (G) Anti-IgG-Fc measuring purified IgG spiked into a BSA solution. (H) Anti-IgM measuring purified IgM spiked into a BSA solution.

Step d. The purpose of this step was to determine the specificity of binding by each antibody on the array using antigen and antibody spike-in experiments. We performed antigen dilution series experiments for a selected set of antigens (Figure 2). Purified antigens at varying concentrations in a serum or BSA background were labeled and incubated on antibody microarrays, and the changes in binding levels were measured at each antibody. The linear response for the antibodies in Figure 2 indicates proper binding of the antigen. The other antibodies on the arrays, which were not specific for the spiked-in antigens, did not show any binding of the antigens, with the exception of the antigen complement C3. We detected measurable binding of C3 at many of the other antibodies on the arrays, indicating that C3 may be non-specifically binding to these antibodies. The level is low and should not affect the quantitation of the data or the ability to detect mid-to-high abundance serum proteins. However, this effect may limit our ability to detect low abundance proteins. A description of how that situation is being addressed appears below.

The spike-in experiments were performed on a limited set of antigens since purified antigens are only available for common proteins and are often very expensive. Also, as described in the previous progress report, we are focusing our validation efforts on the antibodies that provide useful information in the profiling experiments. It will be more efficient to first screen the performance of the antibodies and then perform the validation studies. The validation of the binding specificity can be performed using multiple methods that we now use routinely in the

lab, such as western blots and immunoprecipitations. We expect to do more antibody validations after the next rounds of profiling experiments, which are currently underway. Therefore this aim is ongoing.

Additional progress toward the goals of the project

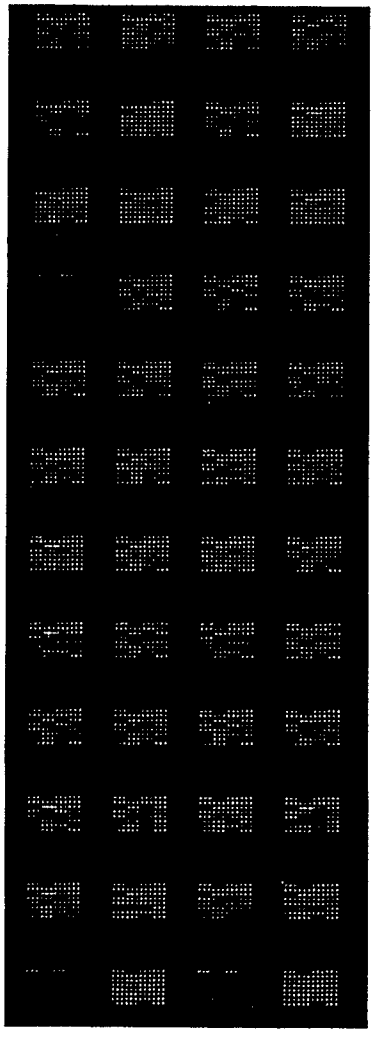


Figure 3. 48 microarrays on a single microscope slide. Each identical microarray was incubated with a different serum sample and detected by TC-RCA.

Collection of additional serum samples. In the first year of the project we obtained serum samples from collaborators Dr. Teh at Baylor College of Medicine and Dr. Partin at Johns Hopkins University. 280 samples were received in the first year. In the second year, to complete our sample set and to allow better statistical analysis of multiplexed data, 200 more samples were obtained from Dr. Partin: another 50 each from patients with benign prostatic hyperplasia, organ-confined prostate cancer, metastatic prostate cancer, and no disease.

Further development of high-throughput sample processing. In year 1 we reported on developments that allowed high-throughput sample processing. This method was important to enhance our ability to run large sample sets. We have further developed those methods. We are now printing 48 microarrays on each microscope slide, and we use our custom-built slide imprinter to imprint hydrophobic wax borders between the arrays, so that a different sample can be isolated on each array. A microscope slide with 48 arrays is shown in Figure 3. We are now routinely using this format. We are in the process of making the sample labeling and preparation steps more high-throughput and automated, as those steps are the current bottleneck in large studies.

Further improvements in the experimental protocol. A major factor that determines the quality of microarray data is the surface upon which the microarrays are printed. Several different types of surfaces have been demonstrated and are commercially available. In year 1, we primarily used nitrocellulose-coated slides (FAST slides, Schleicher & Schuell) since those seemed to perform somewhat better than our previous hydrogel slides (PerkinElmer) and poly-l-lysine-coated slides. In that work we saw evidence that the FAST slide was not optimal for our experiments due to high backgrounds and possible high non-specific binding. Therefore it was important, before continuing with large-scale profiling experiments, to identify the best surface for the experiments.

We performed a systematic comparison of four different surfaces, looking at signal-to-background ratios, reproducibilities, and detection limits. In dilution series of purified antigens in a BSA background, the FAST slides had higher detection limits than the other surfaces (Figure

4). The S/Bs also were lower. The other surfaces performed well, but a slide coated with an ultra-thin layer of nitrocellulose (Path slides, Gentel Biosurfaces) seemed to be the most consistent in all categories. Therefore we began to use the Path slide for subsequent experiments.

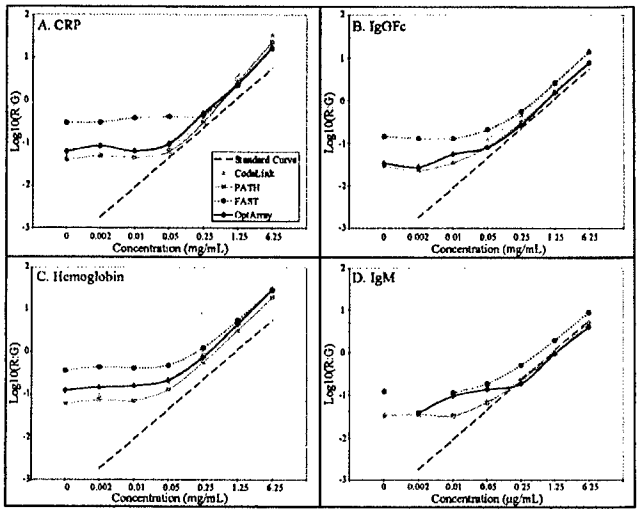


Figure 4. Comparison of surfaces using dilution series. Dilution series of the indicated antigens were performed on four different surfaces (indicated in legend). The signals from the antibodies are shown as a function of the concentrations of the corresponding analytes. The nitrocellulose (FAST) surface had higher detection limits than the other surfaces.

Summary of progress on Task 2

The statement of work for Task 2 is:

Task 2. Collection of antibody microarray protein profiles of prostate cancer serum samples using detection by direct fluorescent labeling and RCA, Months 10-26:

a. Print microarrays as needed using validated antibodies.
b. Perform microarray experiments on each of the serum samples in duplicate using direct labeling and in duplicate using signal amplification by RCA.
c. Prepare data for analysis: "grid" microarray images and reject weak or poor quality data points. Reject data points that do not fall within the linear range of each detection method.

Step a. Over 300 microarrays each containing 96 antibodies were printed in year one for the initial testing studies and the initial data collection on the clinical samples. In year 2, we printed 492 arrays in 3 prints with 91 antibodies per array. The antibodies on the arrays were slightly different between sets 1 & 2 and sets 3 & 4, since sets 3 & 4 included some new antibodies and excluded some antibodies that performed poorly earlier or that were discovered by gel analysis (Figure 1) to not be good. We have also printed many arrays for the additional optimization and development work that is described in this report.

Step b. At the end of year 1, 146 samples had been processed in duplicate on antibody microarrays containing 96 antibodies each. The samples were from patients with organ-confined prostate cancer, non-organ-confined prostate cancer, benign prostatic hyperplasia, and no disease. These experiments were performed on arrays printed on nitrocellulose-coated microscope slides from Schleicher & Schuell, using the TC-RCA detection method. In year 2, we continued the profiling studies initiated in year 1. These arrays, containing 91 different antibodies and printed on our new ultra-thin nitrocellulose surface, were used to profile 150 samples, 50 each from organ-confined cancer, BPH, and healthy, in triplicate experiment sets. We chose to use only three classes instead of four to simplify the analysis in the first stage of

data collection and because we are most interested in detecting early-stage cancer. One of the experiment sets unfortunately failed due to problems running the experiments, leaving us duplicate experiments on this sample set. Thus we currently have four sets of profiling data: two performed on nitrocellulose-coated slides (sets 1 & 2), and two performed on ultra-thin nitrocellulose (sets 3 & 4). The analyses of these data are described under Task 3. All experiments were performed using TC-RCA detection for the reasons described in Task 1.

Step c. This step has been performed for all acquired data. This process is fairly rapid since we have written a software script to automate the process of rejecting low-intensity data points, based on a statistical threshold. Also, we reject data based on a lack of reproducibility between duplicate experiment sets. A 99% confidence threshold in the correlation between the data from the replicate sets is calculated, and data from antibodies not surpassing that threshold are rejected. An example of thresholding the data based on reproducibility is shown in Figure 3. The correlation between the data from the replicate experiment sets was calculated for each antibody, and a histogram of the correlations was generated. Most correlations were in the 0.8-0.9 range, which is highly reproducible. The dashed line gives the approximate threshold for 99% confidence correlation (the threshold varies depending on the number of data points). Antibodies below the threshold were excluded from further analysis. 70 antibodies passed the 99% confidence threshold in sets 1 & 2 and 84 antibodies passed the threshold in sets 3 & 4. An additional data processing step is normalization, which will be performed according to optimized procedures that we described in a recent publication (1). Therefore the data used for the statistical analyses of Task 3 are high quality and reliable.

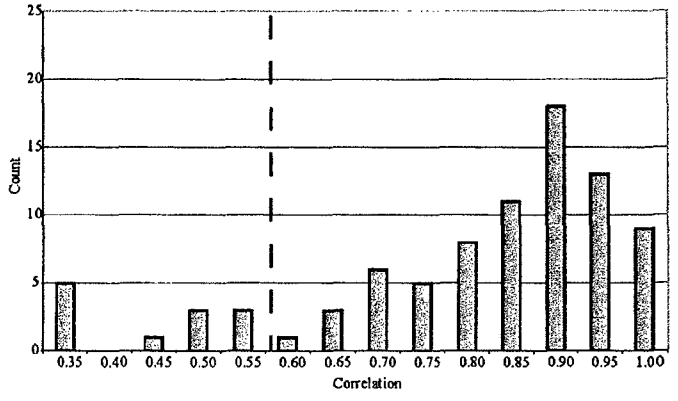


Figure 5. Histogram of correlations between replicate experiment sets. For each antibody, the correlation between the data from sets 3 and 4 was calculated, and a histogram of the numbers of antibodies at each correlation was generated. The dashed line represents the 99% confidence threshold in correlation for most of the antibodies (varies based on number of data points).

Additional progress toward the goals of the project

Reduction of species-specific binding to the antibodies. From our initial analyses of the profiling data, we made the observation that some antibodies seemed to show species-associated binding. Certain groups of antibodies from a common animal of origin showed a common pattern of binding across the samples. For example, most of the antibodies from chicken would have high binding in the same ten samples. Therefore, something in those samples could be recognizing the antibodies based on the species of origin, rather than on the specificities of the antibodies. To further test this hypothesis, we spotted non-specific IgG (acquired from non-immunized animals) from rabbit, mouse, goat, sheep, and chicken, and we observed the correlations in the data from these non-specific antibodies to the data from the specific antibodies of the corresponding animal of origin. The binding patterns from the non-specific antibodies correlated with certain specific

antibodies of the corresponding species, showing the existence of some species-specific binding in the samples.

To counteract this effect, we sought to block the species-specific binding by the addition of the normal, non-specific IgGs to the diluted serum samples. We added varying concentrations of antibodies from chicken, mouse, rabbit, goat and sheep to each of the serum samples and observed the change in binding to spotted antibodies. The binding to the non-specific antibodies was eliminated with the addition of the blocking antibodies in the serum samples (Figure 6A), while the binding to other, specific antibodies was not significantly reduced or altered (Figure 6B).

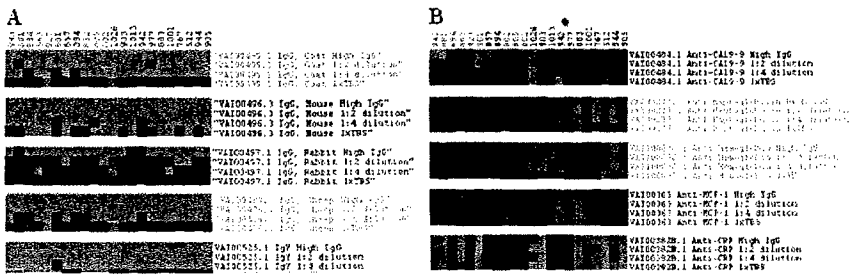


Figure 6. Blocking species-dependent binding. (A) binding to non-specific antibodies from non-immunized antibodies. 23 different serum samples were incubated on arrays containing normal goat (green), mouse (blue), rabbit (red), sheep (aqua), and chicken (pink) antibodies. Each serum sample was diluted into four different buffers: 1X tris-buffered saline (TBS) and three concentrations of IgG blockers. The row labels indicate the antibody and the condition of the incubation, and the squares indicate the signal from the antibodies: green is low signal, red is high signal, and gray is below detection limit. The blockers reduce the non-specific binding to these antibodies to zero. (B) Maintenance of specific signal. The patterns of binding to the other antibodies remained unchanged in response to the blocking buffers.

The addition of the normal-antibody blocker to the serum samples should significantly improve the accuracy and usefulness of the data from some antibodies, and it may reduce low levels of non-specific binding to most of the antibodies. We plan to run the profiling experiments again with the inclusion of the new blocking mix. A manuscript describing this technique is in preparation.

Use of multiplexed sandwich immunoassays for data collection. Another observation we made from previous experiments was that the detection limits of the two-color competitive assays may not be low enough to enable the routine measurements of low-abundance proteins and cytokines in serum. In the two-color assay, all proteins are labeled, and all non-specific binding to the surface or to the antibodies is detected. Low levels of this non-specific binding will negatively affect the detection limit. For example, the non-specific complement C3 binding to many of the antibodies that we observed in the experiments described under Task 1, step d, may have that effect. The two-color assay is very good at accurately and reproducibly detecting changes in mid-to-high abundance proteins, as demonstrated in previous work (2-4), but higher specificity is likely needed to measure low abundance proteins.

The best way to achieve higher specificity is to use a sandwich assay, which uses two antibodies per target—a capture antibody and a detection antibody. To use sandwich assays in a microarray format, multiple capture antibodies are spotted down, unlabeled serum is incubated on the array, and the binding to each antibody is detected using a cocktail of labeled detection antibodies, each antibody corresponding to one of the capture antibodies. Sandwich assays are technically more

difficult than our standard assay, especially in a multiplexed format, so this format will be used for a smaller, select set of targets. We have already developed and validated several assays. For example, we tested several conditions to optimize a microarray assay for HGF (in collaboration with Dr. Brian Cao at VARI) and achieved standard curves with good linearity and detection limits (Figure 7). We are developing a multiplexed sandwich assay for approximately 15 other targets (partly funded through another project). These assays will be used to profile the sample sets in year 3.

Another approach to acquiring sandwich immunoassay data on multiple low-level proteins is to use the Luminex bead-based platform. The Luminex system enables multiplexed detection of up to around 30 different analytes, and kits have been developed for many different analytes. A collaborator at the University of Pittsburgh Medical Center, Dr. Anna Lokshin, directs a Luminex core facility. She has agreed to a collaboration to run some of the samples for this project on her system. The analytes to be measured are shown in Table 1. We have preliminary data from 150 samples: 50 each from organ-confined prostate cancer, BPH, and healthy controls. We are evaluating and analyzing the data now to decide whether to pursue the use of this platform further.

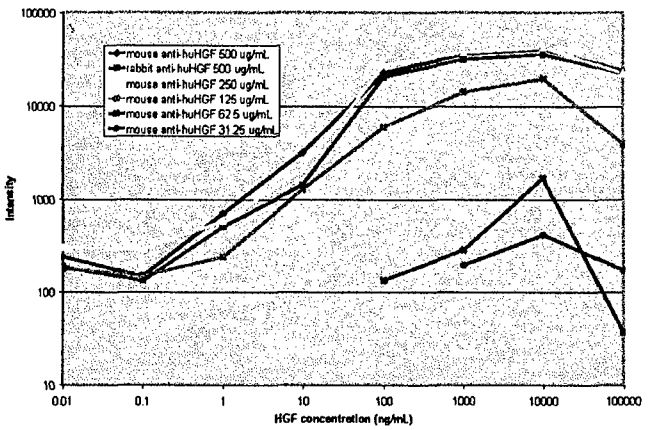


Figure 7. Development of a microarray sandwich assay for HGF. Several different conditions were tested (indicated in the legend) to optimize the assay for HGF, shown by the standard curves.

Summary of progress on Task 3

The statement of work for Task 3 is:

Task 3. Final analysis of data, Months 27-36:

a. Correlation of individual proteins and combinations of proteins with grade, stage, extent of disease and follow-up information to identify markers of aggressive disease.
b. Correlation of proteins with each other, with functional groups of proteins and with clinical information to infer functional relationships between individual proteins, host functions, and the cancer.

These items are scheduled to begin in the third year, but we have begun them in the second year to learn more about the data we are generating and to identify possible changes we need to make in acquiring additional data. For example, our initial analyses identified the species-specific binding that was described under Task 2. Our initial data sets and analyses are also identifying

the antibodies that give valuable information about the samples. These antibodies will be used in subsequent data sets and will be further validated using other methods.

The initial analysis consisted of identifying antibodies with binding profiles that were statistically different between the sample groups. Using the data from sets 1 & 2, t-tests were performed on the data from each antibody to evaluate the differences between the patient classes (organ-confined prostate cancer, non-organ-confined prostate cancer, BPH, and healthy). The individual sets 1 & 2 and the averaged data from the two sets was analyzed. Several antibodies showed differences between the groups (Table 2). Many of the differences had been previously observed or were consistent with literature reports. For example, we previously observed elevated von Willebrand factor, elevated alpha1-antichymotrypsin, and reduced immunoglobulin in prostate cancer (3). Reduced alpha-2-macroglobulin, elevated HGF, and higher IL-8 also have been observed. The elevated HGF is particularly interesting, since it is a ligand for Met receptor, which is important for driving prostate cancer metastasis to bone. We will use our sandwich assay to confirm and further explore that finding. Alpha-2-macroglobulin and alpha-1-antichymotrypsin both bind and inhibit prostate-specific antigen, and the measurement of those molecules together along with PSA could be valuable in multiparametric classification algorithms. Other molecules could provide insights into the differences between benign disease and cancer, since several antibodies showed binding that was elevated or repressed specifically in the BPH samples.

Abbreviation	Analyte
DR5	Death receptor 5
EGF	Epidermal growth factor
EOTAXIN	Eotaxin
FGFB	Fibroblast growth factor b
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HGF	Hepatocyte growth factor
IFNa	Interferon alpha
IFNg	Interferon gamma
IL-1a	Interleukin 1a
IL-1b	Interleukin 1b
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12 p40	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IP10	Interferon inducible protein 10
MCP-1	Monocyte chemotactic protein 1
MIG	Monokine induced by gamma-inferferon
MIP-1a	Macrophage inflammatory protein 1 alpha
MIP-1b	Macrophage inflammatory protein 1 beta
RANTES	regulated upon activation, normal T-cell expressed, and presumably secreted - CCL5
TNFa	Tumor necrosis factor a
TNFR1	Tumor necrosis factor receptor
TNFRII	Tumor necrosis factor receptor
VEGF	Vascular endothelial growth factor

Table 1. Analytes included in the Luminex 33-plex assay at UPMC.

As we complete and improve our data sets, we will further explore the most provocative of these findings to complete the goals of Task 3. Additional analyses will include the use of multiparametric methods to classify the samples based on combinations of measurements (as opposed to individual measurements) the exploration of correlations between groups of antibodies and the patient classes (to identify possible co-regulation or functional relationships). Our next data sets will be collected using antibodies that have produced reproducible and informative results in the first data sets, and they will be performed using our improved methods. In addition, we will collect data using sandwich immunoassays for a selected set of antigens. Those data will be combined with the data from the two-color assays. The binding specificities of the important antibodies in the analyses will be characterized and validated. With the technical improvements that we have made to the assays, we expect to achieve high quality and informative data.

Antibody	Metastic vs Healthy		OC vs Healthy		All Cancer vs Healthy		OC vs Benign		Metastic vs Benign		Benign vs Healthy	
	T Test average	Higher	T Test average	Higher	T Test average	Higher	T Test average	Higher	T Test average	Higher	T Test average	Higher
VAI00053 Anti-Angiostatin (ab-2)	0.0313	M	0.0063	OC	0.0047	C	---	---	---	---	0.0295	H
VAI00194 Anti-Von Willebrand Factor	0.0352	M	---	---	0.0181	C	---	---	---	---	0.0127	B
VAI00244 Anti-IGG1	0.0109	H	0.0087	H	0.0169	H	---	---	---	---	0.0110	H
VAI00261 Anti-VEGF	---	---	0.0113	OC	---	---	---	---	---	---	---	---
VAI00263.1 Anti-Alpha 2-macroglobulin	---	---	0.0217	H	0.0139	H	---	---	---	---	---	---
VAI00269-2 Anti-HGF (MabA 7)	---	---	0.0136	OC	---	---	---	---	---	---	---	---
VAI00290-2 Anti-HGF (Polyclonal)	0.0359	M	0.0182	OC	0.0065	C	---	---	---	---	0.0144	B
VAI00308-2 Anti-TSP-1 (Ab-2)	---	---	---	---	---	---	---	---	0.0159	B	---	---
VAI00335.1 Anti-Gelsolin	0.0154	M	---	---	0.0187	C	---	---	---	---	---	---
VAI00336 Anti-Caveolin-1	---	---	---	---	---	---	---	---	---	---	0.0365	B
VAI00348 Anti-b2-Microglobulin	---	---	0.0170	OC	0.0044	C	---	---	---	---	0.0191	B
VAI00369 Anti-Lactate dehydrogenase 5 (LD5)	---	---	0.0046	H	0.0123	H	---	---	---	---	0.0056	H
VAI00422.1 Anti-Ferritin, Liver	---	---	---	---	---	---	---	---	0.0048	B	0.0234	B
VAI00435.1 Anti-CD106 (VCAM-1, INCAM-110)	---	---	---	---	---	---	---	---	0.0206	C	0.0144	H
VAI00441.1 Anti-Pim 1	---	---	0.0450	H	---	---	---	---	0.0123	C	0.0175	H
VAI00445 Anti-CD11a (LFA-1, Integrin aL)	---	---	---	---	---	---	---	---	0.0251	C	---	---
VAI00447 Anti-trypsin	---	---	---	---	---	---	0.0120	B	---	---	---	---
VAI00448 Anti-IL-8	---	---	---	---	0.0383	C	---	---	---	---	0.0094	B
VAI00460 Anti-MIP-1alpha	---	---	0.0004	OC	0.0021	C	---	---	---	---	0.0096	B
VAI00464 Anti-Cadherin E	---	---	0.0075	OC	0.0110	C	---	---	---	---	0.0012	B
VAI00465 Anti IL-1beta (affinity pure)	---	---	---	---	---	---	---	---	---	---	0.0004	B
VAI00467 Anti-IL-6	---	---	0.0057	OC	0.0303	C	---	---	---	---	0.0272	B
VAI00468 Anti-IL-10	---	---	0.0213	OC	0.0161	C	---	---	---	---	0.0040	B
VAI00469 Anti-laminin	---	---	0.0419	OC	---	---	---	---	0.0188	B	0.0127	B
VAI00470 Anti-MMP1	---	---	0.0159	OC	0.0126	C	---	---	---	---	0.0012	B
VAI01032 Anti-Alpha1 Antichymotrypsin	---	---	0.0034	OC	---	---	---	---	---	---	---	---
VAI10003 Anti-IgG-Fc	---	---	0.0318	H	0.0365	H	---	---	---	---	---	---
VAI10007 Anti-Hemoglobin	---	---	---	---	---	---	---	---	---	---	0.0342	H
VAI10011 Anti-IgA	---	---	0.0349	H	0.0288	H	---	---	---	---	0.0214	H

KEY RESEARCH ACCOMPLISHMENTS

- Further development and improvement of the methods. We instituted improved quality control for the antibodies, improved our ability to run samples in high-throughput, and improved the data quality through the use of a better-performing surface.
- Identified the existence of species-dependent, non-specific binding and developed methods to block that effect. We showed that the inclusion of immunoglobulin blockers in the serum dilution buffer removes non-specific, species-dependent binding while maintaining normal levels of specific binding.
- Pursued an alternate strategy to allow more specific and sensitive detection of a select set of analytes. Multiplexed sandwich immunoassays are being developed in-house for 15 analytes, and data from a commercial platform providing analysis of 33 analytes is being evaluated.
- Completed replicate profiling of multiple analytes on sets of serum samples from prostate cancer patients and controls.
- Identified many antibodies with statistically different levels between the patient groups, many previously observed and some new.

REPORTABLE OUTCOMES

- Funding was obtained based on research supported by this award: R21 RFA-CA-05-003. A project to explore the use of longitudinal measurement of multiple markers for the improved diagnostics of prostate cancer was funded by the NCI's Innovative Molecular Analysis Technologies program. That project is built upon some of the developments from this project and will also complement the findings. Start date is June 1, 2005.
- Presentations (where portions of the results from this project were presented): American Chemical Society's "Advanced Microarray Strategies for Biopharmaceuticals" conference, Boston, June 8, 2004; CHI's "Protein Biomarkers," September 9, 2004; Michigan Prostate Cancer Colloquium, April 23, 2005.
- Posters: American Association for Cancer Research annual meeting, April 17, 2005; VARI annual retreat, April 28, 2005.

CONCLUSIONS

Significant progress toward the goals of this project was achieved. We completed the experiments and analysis to determine the best method for running the assays and have used that method to profile a large sample set several times. We greatly improved our methods to allow the acquisition of higher quality data in a more high-throughput fashion. Further, some limitations to the existing assay were identified through our initial analyses. One limitation, species-specific binding to the antibodies, was eliminated through the use of novel blocking agents. Another limitation, limited sensitivity caused by low-level non-specific binding of molecules such as complement C3, is being addressed through the use of a complementary alternate strategy—multiplexed sandwich assays—for a limited number of low-abundance analytes. The initial analyses of the profiling data achieved in years 1 and 2 have revealed provocative results on the differences between the sample groups. These results will be supplemented and verified in studies of year 3. The ongoing profiling studies are being performed with our improved technology and new blocking agents. That data will be joined with the data from the sandwich assays. The combined data sets will be analyzed in year 3 to achieve the aims of task 3. Publications on the novel blocking strategy and on the evaluation of the four different surfaces are in preparation, and a manuscript on the profiling studies of the prostate cancer sera should be submitted this year. In summary, the work so far has resulted in a better understanding of the performance of antibody microarrays, significant improvements to the methods, and preliminary indications of some of the major differences between sera from prostate cancer, benign disease, and healthy controls. This work is on target to complete the aims of the proposal by the end of year 3.

REFERENCES

1. Hamelinck, D., Zhou, H., Li, L., Verweij, C., Dillon, D., Feng, Z., Costa, J., and Haab, B. B. Optimized normalization for antibody microarrays and application to serum-protein profiling. Mol Cell Proteomics, 2005.
2. Haab, B. B., Dunham, M. J., and Brown, P. O. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biology, *2:* 1-13, 2001.
3. Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R., Burke, J., Butler, E. B., Teh, B. S., and Haab, B. B. Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. Proteomics, *3:* 56-63, 2003.
4. Zhou, H., Bouwman, K., Schotanus, M., Verweij, C., Marrero, J. A., Dillon, D., Costa, J., Lizardi, P. M., and Haab, B. B. Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-protein measurements. Genome Biology, *5:* R28, 2004.

Principal Investigator: Haab, Brian B.

AACR 2005 and Michigan Prostate Cancer Colloquium abstract, April 2005

Prostate-cancer-associated immunoreactivity identified using arrays of tumor-derived proteins
Brian B. Haab, Sara Forrester, Ji Qiu, Leslie Mangold, Alan Partin, David Misek, Samir Hanash, Gilbert S. Omenn

Broad characterization of the immune responses elicited by tumors has valuable potential applications in diagnostics and in basic research. We present here the use of microarrays of tumor-derived proteins to profile the antibody repertoire in the sera of prostate cancer patients and controls. Rotofor isoelectric focusing and reversed-phase HPLC (20 x 88 fractions) were used to separate proteins from the prostate cancer cell line LNCaP into 1760 fractions, which were spotted in microarrays on coated microscope slides. The microarrays were incubated with serum samples from 51 men with prostate cancer and 46 male controls, in duplicate. The amount of bound immunoglobulin from each serum sample at each fraction was quantified by incubating biotinylated anti-human Ig on the microarrays, followed by Cy3-labeled streptavidin. Multiple fractions had significantly and reproducibly higher levels of immunoglobulin binding in the prostate cancer samples compared with controls. 62 fractions had high antibody reactivity in at least five of the cancer patients but no more than one of the control patients. Many of the 62 fractions likely represent the same protein, since they appeared in distinct clusters in the fractionation. The level of immunoreactivity varied between patients, ranging from some patients with no reactivity to others with broad reactivity to many proteins. The breadth of the immune response among the cancer patients did not correlate with PSA level, Gleason grade, or stage of disease. Western blots, immunoprecipitations, and LC-MS methods are being used to identify the immunoreactive proteins. Identification and isolation of these proteins will facilitate more precise characterization of the specificity and prevalence of the immune responses in cancer patients. A complementary approach using antibody microarrays to detect serum protein profiles is being combined with these antigen microarrays to enhance tumor antigen and auto-antibody discovery for prostate cancer diagnosis. Microarrays of fractionated proteins could be a powerful tool for tumor antigen discovery and cancer diagnosis. Supported by Michigan Economic Development Corporation grant GR356 and Department of Defense grant 17-03-1-0044.

Protein Biomarkers abstract, September 2004

Antibody microarrays and two-color rolling circle amplification for probing cancer-associated serum protein profiles

The profiling of proteins in biological samples using antibody microarrays has the potential to yield new discoveries. In a step toward further enabling that capability, we developed the use of two-color rolling-circle amplification (Two-Color RCA) to measure the relative levels of proteins from two serum samples that have been captured on antibody microarrays. We are using this tool to study the associations of many circulating tumor and host response markers with a variety of cancers. The presentation will cover the performance and optimal use of the technology, in addition to the biological significance and potential clinical usefulness of the observed protein alterations in the sera of cancer patients.

Principal Investigator: Haab, Brian B.

American Chemical Society abstract, June 2004

Antibody microarrays enable highly multiplexed and rapid protein measurements in low sample volumes. The profiling of proteins in sera and other bodily fluids using this tool should offer new opportunities for biomarker discovery and insights into disease biology. Robotically spotted microarrays of antibodies and proteins were used to measure the relative abundances of multiple proteins in serum samples from prostate cancer and pancreatic cancer patients and controls. Serum proteins that had been coupled to either a fluorescent tag (e.g. Cy3) or a hapten (e.g. biotin) were incubated on the microarrays, and specific proteins bound to the immobilized molecules on the microarrays through specific interactions. After washing away unbound proteins, bound proteins were detected using the fluorescent tag or amplified signal (using rolling circle amplification, RCA) from the hapten-labeled proteins. RCA significantly enhanced detection sensitivity while maintaining the accuracy and precision of the measurements. Measurements of dozens of proteins in sera from cancer patients and controls revealed significant differences in protein abundances between the two sample groups. The biological significance and potential clinical usefulness of the observed protein alterations in the sera of cancer patients will be discussed.